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Evaluation of the zoonotic potential of multiple subgroups of clade 2.3.4.4 influenza A (H5N8) virus

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ABSTRACT

Clade 2.3.4.4 H5N8 highly pathogenic avian influenza viruses (HPAIVs) have spread worldwide. Phylogenetic analysis identified two genetic groups of the H5N8 HPAIVs in South Korea; group A evolved further into four subgroups. Here, we examined the zoonotic potential, both in vivo and in vitro, of genetically distinct subgroups of H5N8 HPAIVs isolated in South Korea. When compared with other subgroups, A/mallard/Korea/H2102/2015 (H2102) virus caused relatively severe disease in mice at high doses. In ferrets, all H5N8 viruses replicated restrictively in the respiratory tract and did not induce significant clinical signs of influenza infection. *In vitro* studies, all viruses displayed a hemagglutinin phenotype that was poorly adapted for infection of mammals, although the H2102 virus exhibited higher replication kinetics at 33 °C than the others. Although H5N8 HPAIVs have not yet acquired all the characteristics required for adaptation to mammals, their ability to evolve continuously underscores the need for timely risk assessment.

1. Introduction

Since they were first detected, H5N1 highly pathogenic avian influenza viruses (HPAIVs) belonging to the A/Goose/Guangdong/1/1996 (GsGd) lineage have become panzootic in domestic poultry in Eurasia and Africa. Highly pathogenic avian influenza (HPAI) H5N1 viruses have evolved into multiple phylogenetic clades based on the hemagglutinin (HA) gene, and pose a threat to public health (Eagles et al., 2009). World Health Organization data show that HPAI H5N1 viruses are responsible for more than 850 human infections and 440 deaths (approximately 53% mortality) worldwide, primarily in 16 countries (Programme, 2016).

H5N8 HPAIVs belonging to H5 clade 2.3.4.4 were first isolated on duck farms in eastern China in 2010 (Zhao et al., 2013). In 2013, novel reassortant H5N8 viruses were isolated in live poultry markets in eastern China, and subsequently in poultry and wild birds in the Republic of Korea and Japan (Kanehira et al., 2015; Lee et al., 2014; Wu et al., 2014). Phylogenetic analysis identified two distinct genetic groups of H5N8 HPAIVs in the Republic of Korea: group A (A/broiler duck/Korea/Buan2/2014-like) and group B (A/breeder duck/Korea/Gochang1/2014-like). In late 2014, HPAI H5N8 viruses were also discovered in Europe and North America, and were then reintroduced into South Korea and Japan (Hill et al., 2015; Ip et al., 2015; Kwon et al., 2016; Verhagen et al., 2015). The dominant viruses belonging to group A were further classified into four distinct subgroups: C1 (South Korea), C2 (South Korea/Japan), C3 (North America/Japan), and C4 (Europe/Japan/South Korea) (Hill et al., 2015; Song et al., 2017).

To date, there are no known human cases of infection with H5N8 HPAIVs. However, since cases of human infection with H5N6 HPAIVs belonging to clade 2.3.4.4 have been reported in China (Pan et al., 2016; Yang et al., 2015), it is important to assess their zoonotic capability and potential threat to public health. Early isolates (group A and B) from the Republic of Korea (Kim et al., 2015), the Dutch H5N8 HPAIV (A/Chicken/NL/EMC-3/2014) representing subgroup C4 (Richard et al., 2015), and the North American H5N8 HPAIV (A/Gyrfalcon/Washington/41088-6/2014) representing the C3 subgroup (Pulit-Penaloza et al., 2015) show low-to-moderate virulence in ferrets.

Most published studies examining the zoonotic potential of H5N8 HPAIVs focused on early isolates and were limited to a single subgroup. However, the worldwide distribution and genetic divergence of these viruses necessitates continued investigation (Saito et al., 2015). Here, we examined the genetic characteristics, pathogenicity (in mice and ferrets), receptor binding properties, HA acid stability, and replication kinetics (in human bronchial epithelial cells) of three genetically

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Table 1
Molecular characteristics associated with viral pathogenicity.

Virus name (H5N8)	Genetic subgroup	HA[a]			M1			M2	NS1	PB2							PB1	PB1-F2
		Cleavage site	133	156	15	30	215	31	42	89	309	477	495	627	676	701	317	66
H1731	C1	RERRRKR↓GLF	A	A	I	D	A	N	S	V	D	G	V	E	T	D	I	S
H1924	C4	RERRRKR↓GLF	A	A	I	D	A	N	S	V	D	G	V	E	T	D	I	S
H2102	C2	RERRRKR↓GLF	A	A	I	D	A	N	S	V	D	G	V	E	T	D	I	S
Gyr/WA	C3	RERRRKR↓GLF	A	A	I	D	A	N	S	V	D	G	V	E	T	D	I	S
Gochang1	B	REKRRKR↓GLF	A	A	I	D	A	S	S	V	D	G	V	E	T	D	M	–
Buan2	A	RERRRKR↓GLF	A	A	I	D	A	N	**S**	V	D	G	V	E	T	D	I	S

[a] H5 numbering was used. Abbreviations: HA, hemagglutinin; NA, neuraminidase; PB, polymerase basic; M, matrix; NS, nonstructural; H1731, A/broiler duck/Korea/H1731/2014; H1924, A/domestic mallard duck/Korea/H1924/2014; H2102, A/mallard duck/Korea/H2102/2015; Gyr/WA, A/Gyrfalcon/Washington/41088-6/2014; Gochang1, A/breeder duck/Korea/Gochang1/2014; Buan2, A/duck/Korea/Buan2/2014.

distinct subgroups (C1, C2, and C4) of H5N8 HPAIVs isolated in South Korea in the winter of 2014–2015.

2. Results

2.1. Genetic characterization of multiple subgroups of H5N8 HPAIVs

Hill et al. (2015) categorized HPAI H5N8 viruses belonging to group A (identified during the winter of 2014–2015) into four genetically distinct subgroups. Therefore, we investigated the known mammalian-adapting substitutions present in the sequences of the HPAI H5N8 viruses. We found 14 amino acid substitutions in the HPAI H5N8 sequences from all genetic subgroups except group B; these substitutions may affect receptor binding preference, virulence, and replication in mammals (Table 1). We did not detect any well-known mammalian adaptive markers (Hatta et al., 2001), such as E627K and D701N in PB2, in any of these representative viruses. However, five amino acid substitutions in PB2 (L89V, G309D, R477G, I495V, and A676T), which can compensate for the effect caused by the E627K substitution in mice (Li et al., 2009), were identified in all of these H5N8 viruses. Two of these substitutions, S133A and T156A in HA (numbering based on H5 A/Vietnam/1203/2004), increase affinity for α2,6-linked sialic acid receptors (Herfst et al., 2012; Yang et al., 2007). Other substitutions (V15I, N30D, T215A in M1; P42S in NS1; M317I in PB1; and N66S in PB1-F2) increase virulence in mammals (Fan et al., 2009; Gao et al., 2009; Jiao et al., 2008; Katz et al., 2000). A molecular marker of amantadine resistance was detected in the matrix-2 protein sequences of all H5N8 HPAIVs except a representative virus (A/breeder duck/Korea/Gochang1/2014) from genetic group B. Moreover, we compared the sequences of representative HPAI H5N8 viruses and found several amino acid differences among the subgroups (Supplementary Table 1).

2.2. Pathogenicity of H5N8 viruses in mice

To assess the effects of genetic evolution on the pathogenicity of clade 2.3.4.4 H5N8 HPAIVs in mice, groups of mice were intranasally inoculated with 10^6 EID_{50} of each H5N8 virus. The survival rates of the mice inoculated with A/broiler duck/Korea/H1731/2014 (H5N8, abbreviated as H1731), A/domestic mallard duck/Korea/H1924/2014 (H5N8, abbreviated as H1924), and A/mallard duck/Korea/H2102/2015 (H5N8, abbreviated as H2102) viruses were 80%, 100%, and 20%, respectively (Fig. 1A). Mice inoculated with the H1731 (C1) or H1924 (C4) viruses showed no weight loss or obvious signs of disease, apart from one mouse infected with the H1731 virus (this mouse was euthanized due to paralysis on Day 13 post-infection (p.i.)) (Fig. 1B). By contrast, mice infected with the H2102 (C2) virus showed significant weight loss ($p < 0.001$) when compared with mice in other groups. Moreover, severe neurological symptoms were observed in one or two out of five mice in each group infected with the H2102 virus at titers $\geq 10^4$ EID_{50} (data not shown).

Regardless of genetic subgroup, all of the H5N8 viruses tested replicated in mouse lung without prior adaptation (Table 2). Virus titers in the lungs of mice infected with the H1731 and H2102 viruses were 5.0 ± 0.4 $\log_{10}$ and 5.1 ± 0.4 $\log_{10}$, respectively, on Day 3 p.i., and were significantly higher than those in mice infected with the H1924 virus ($p < 0.01$). By contrast, mice infected with the H1924 virus showed the lowest viral titer in the lung (2.8 ± 0.8 $\log_{10}$). Extra-pulmonary dissemination of H5N8 viruses was restricted exclusively to the spleen. The H2102 virus had a 50% mouse lethal dose (MLD_{50}) of $10^{5.3}$ EID_{50}, whereas the H1731 and H1924 viruses had MLD_{50} titers of $10^{6.5}$ EID_{50} and $> 10^7$ EID_{50}, respectively. While the H1731 and H1924 viruses were considered to be of low pathogenicity, the H2102 virus was moderately pathogenic in mice (based on previously described criteria) (Maines et al., 2005).

2.3. Pathogenesis of H5N8 HPAIVs in ferrets

Ferrets are an excellent animal model for studying the pathogenesis of influenza viruses in mammalian hosts. Groups of six ferrets were intranasally inoculated with 10^7 EID_{50} of H1731, H1924, and H2102 viruses and observed for signs of infection. None of the ferrets showed any mortality or significant morbidity (Fig. 2B). Ferrets infected with the H2102 (C2) virus showed mild respiratory signs such as sneezing, whereas other groups showed no pronounced respiratory symptoms. At Days 1 and 3 p.i., the body temperature of H1731 (C1)- or H2102-infected ferrets was significantly higher than that of ferrets infected with the H1924 virus (C4) ($p < 0.01$, Fig. 2A). In all groups, viruses were shed in nasal washes at titers ranging from $10^{2.5}$ to $10^{3.5}$ EID_{50} at Day 7 p.i. (Table 3). On Day 3 p.i., H1731 and H2102 viruses were detected in nasal turbinate from all inoculated ferrets, and in the lungs of two inoculated ferrets, at titers ranging from $10^{1.6}$ to $10^{2.7}$ EID_{50}. No virus was detected in tissues outside the respiratory tract of H5N8 HPAIV-inoculated ferrets. A hemagglutination inhibition (HI) assay revealed seroconversion in all but one of the H1731-infected ferrets at Day 14 p.i.

2.4. Receptor binding specificity and acid stability of the HA protein of H5N8 HPAIVs

We performed a solid-phase direct binding assay with α2,3 and α2,6 sialylglycopolymers to determine the affinity and specificity of three genetically distinct subgroups of HPAI H5N8 viruses (Fig. 3). The control A/California/04/2009 (H1N1, abbreviated as CA09) virus showed a selective binding preference for α2,6-linked sialic acids (α2,6-SAs). By contrast, all representative HPAI H5N8 viruses preferentially bound to α2,3-SAs, with minimal binding to α2,6-SAs, indicating that these viruses retain affinity for avian-like α2,3-SA receptors.

After entry of the virus particle into host cells, HA protein trimers undergo irreversible conformational changes in the acidic environment of the endosome; these changes promote membrane fusion (Skehel and